

# Monitoring of exposure to acrylonitrile by determination of N-(2-cyanoethyl)valine at the N-terminal position of haemoglobin

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A selected ion monitoring gas chromatography-mass spectrometry (GC-MS) procedure was developed to determine the interaction product formed by acrylonitrile (ACN) with the N-terminal amino group in haemoglobin. The product, N-(2-cyanoethyl)valine (CEV), was analysed following its release from the protein by a modified Edman degradation procedure. Quantitation was achieved using N-(2-cyanoethyl)-[<sup>2</sup>H<sub>5</sub>]Val-Leu-Ser as internal standard. The limit of detection of the assay was 1 pmol CEV/g globin. A close to linear dose-response relationship was found for adduct formation in rats treated with ACN by gavage. On the basis of a linear extrapolation, a dose of 1 mg/kg body wt yielded 248 pmol CEV/g globin. Two groups of workers who were exposed to ACN contained 1984 ± SD 2066 (n = 9) and 2276 ± SD 1338 (n = 7) pmol CEV/g globin respectively. These values were highly significantly greater (P < 0.01 following a one-way analysis of variance with a logarithmic transformation of the data) than those in a group of control workers in the same factory (31.1 ± SD 18.5 pmol CEV/g globin, n = 11). The concentrations of N-terminal CEV in globin samples from 13 smoking and 10 non-smoking mothers and from their newborns were determined. Adduct levels in the smokers averaged 217 ± 85.1 pmol CEV/g globin, significantly higher than the levels in non-smokers, which were undetectable. Individual values in the mothers were very highly correlated with the levels in their babies (which averaged 99.5 ± 53.8 pmol CEV/g globin), which demonstrates that transplacental transfer of ACN occurs. Significant correlations were also found between the number of cigarettes smoked per day by the mother and the CEV levels in both the mothers' and newborns' globin. There was, however, no correlation between the CEV levels and those of the ethylene oxide adduct N-(2-hydroxyethyl)valine in samples from either the mothers or babies.

\*Abbreviations: ACN, acrylonitrile; CEO, 2-cyanoethylene oxide; PFPITC, pentafluorophenylisothiocyanate; PFPTH, pentafluorophenylthiohydantoin; GC-MS, gas chromatography-mass spectrometry; CEV, N-(2-cyanoethyl)valine; MTBSTFA, N-methyl-N-t-butyl(dimethylsilyl)trifluoroacetamide; TBDMCS, t-butyl-dimethylchlorosilane; TBDMSI, t-butyl-(dimethylsilyl); [<sup>2</sup>H<sub>5</sub>]CEV, N-(2-cyanoethyl)-[<sup>2</sup>H<sub>5</sub>]valine; CP, continuous polymerization workers; MM, maintenance mechanics; HEV, N-(2-hydroxyethyl)valine.

## Introduction

Acrylonitrile (ACN\*) is widely used in industry for the manufacture of plastics and synthetic fibres, resulting in considerable human exposure to this chemical. ACN is also a component of tobacco smoke, which represents a further route of human exposure (1).

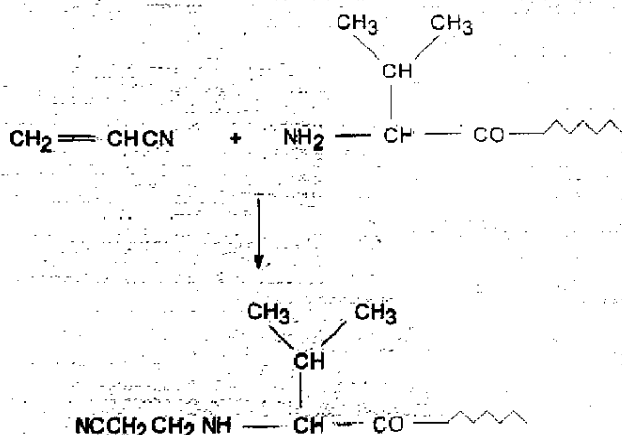
Administration of ACN to rats by inhalation, gavage or addition to the drinking water has produced tumours of the brain, stomach and Zymbal glands (2,3). Carcinogenicity in other animal species has not yet been proven. Evidence for the carcinogenicity of ACN in humans is still limited owing to: (i) the insufficient length of time that has elapsed since initial exposures; (ii) the small working populations that have been studied; (iii) methodological difficulties; (iv) multiple exposures; (v) the confounding effects of smoking habit (4). From the human epidemiological studies that have been conducted on ACN-exposed workers, some have shown statistically significant increases in the incidence of lung and prostate cancers (5,6) and others raise concern about the possible association between ACN exposure and excess lymphatic and stomach cancer incidence (7). On the basis of the available epidemiological and experimental data, an IARC working group has considered ACN as possibly carcinogenic to humans (8).

Most studies on the metabolism and distribution of ACN have been performed in the rat. *In vitro* experiments carried out in rat hepatocytes, liver microsomes, lung microsomes and lung cells (9) have shown that ACN can be metabolized by cytochrome P450-mediated oxidation to an epoxide, 2-cyanoethylene oxide (CEO). Liver microsomes produced more CEO than lung microsomes, suggesting that epoxidation occurs mainly in the liver (10,11). CEO formation has also been observed following incubation of ACN with microsomes from mice and humans (11). ACN and CEO can undergo direct conjugation with glutathione (9,12), which seems to be the main detoxification pathway and leads to the excretion of N-acetyl derivatives and other catabolic products (13-15).

ACN and CEO are electrophilic and can bind irreversibly to proteins and nucleic acids (12,16,17), forming adducts. Haemoglobin adducts have been extensively used as markers of exposure to electrophilic carcinogens (18). Monitoring such adducts offers a relevant measure of extent of exposure to a carcinogen over an extended period owing to the long lifetime of red blood cells and the absence of adduct repair. Haemoglobin is also available from blood in large amounts, which is an important practical advantage when compared with the analysis of DNA adducts. Characterization and quantitative determination of the adducts formed in human haemoglobin by ACN and CEO would add information (which is currently lacking) on exposure to, and metabolism of, ACN in humans.

There are several sites in haemoglobin susceptible to electrophilic attack. Adducted sites that have been used for biomonitoring purposes include the sulphhydryl in cysteine, the imidazole in histidine, carboxylic acid groups in aspartic and

glutamic acids and the amino group in N-terminal valine (18). Adducts at N-terminal valine have had extensive use as biological monitors since the development by Törnqvist *et al.* (19) of a practical method for selectively cleaving alkylated N-terminal valine from the protein chain. This method is a modified Edman degradation procedure and involves coupling the alkylated valine with pentafluorophenylisothiocyanate (PFPITC) under mildly basic conditions to produce a pentafluorophenylthiohydantoin (PFPTH) derivative which can be analysed by gas chromatography-mass spectrometry (GC-MS). The adduct formed by ACN at the N-terminal valine in haemoglobin is formed by an addition mechanism, generating *N*-(2-cyanoethyl)valine (CEV) (Scheme 1).



Scheme 1. Reaction of ACN with N-terminal valine in haemoglobin.

Osterman-Golkar *et al.* (20) have reported on the use of the modified Edman degradation technique to determine the levels of CEV in globin of rats treated with ACN in their drinking water and also in eight humans (four smokers and four non-smokers). N-Terminal CEV has also been measured in globin of 41 workers in the People's Republic of China using a similar analytical approach (21). In our present work, we have applied an analogous (but experimentally different) approach to determine N-terminal CEV in the globin of three populations exposed to ACN: a group of workers involved in the production of acrylic fibres, a population of smoking mothers and their newborn babies and rats injected with doses ranging from 1 to 10 mg/kg.

An essential step in quantitation of protein adducts is the synthesis and characterization of the appropriate standards, i.e. a solution with an accurately known amount of the adduct to be determined, and an internal standard, preferably containing the same adduct labelled with a stable isotope. The optimal conditions for the modified Edman degradation procedure are different for the free adducted amino acid compared with when the amino acid is linked within a peptide (20). For this reason, the use of free adducted amino acids as standards for the quantitation of adducts attached to globin may not be reliable (22). The ideal standards would be globin modified with the carcinogen and globin modified with a stable isotope-labelled carcinogen. However, the accurate determination of the amount of adduct in *in vitro* labelled globins is a complicated and time consuming process. One good way to overcome these difficulties is to use an adducted peptide whose adduct content could be easily determined and which should behave in a similar

way to the globin during the modified Edman degradation procedure.

For this purpose, we have synthesized the adduct of ACN with the N-terminal tripeptide of the  $\alpha$ -chain of human haemoglobin (Val-Leu-Ser), to be used as internal standard in the quantitation of CEV in the sample haemoglobins (23). The valine used for synthesis of the tripeptide was either unlabelled or isotopically labelled with eight atoms of deuterium. This approach of ours differs from that of Osterman-Golkar *et al.* (20) and Bergmark *et al.* (21), who used the adduct of  $[^2\text{H}_3]\text{ACN}$  with valylvalylglycine and the adduct of  $[^3\text{H}_2]$ -ethylene oxide with globin as internal standards respectively.

## Materials and methods

### Chemicals

ACN was supplied by Aldrich (Gillingham, UK). *N*-Methyl-*N*-(4-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% *t*-butyldimethylchlorosilane (TBDMCS) was purchased from Pierce and Warriner (Chester, UK). PFPITC was obtained from Fluka Chemicals (Dorset, UK). Formamide (Analaar, Fluka Chemicals, Dorset, UK) was purified by elution through aluminium oxide (activity grade 1, Woelm). All other solvents were of anar grade and were redistilled before use. Sep-Pak  $\text{C}_{18}$  cartridges (Millipore, Croyley Green, UK) were washed successively with ethyl acetate (4 ml), methanol (4 ml) and water (4 ml) before use.

CEV was prepared by cyanoethylation of *N*-valine with ACN using an equivalent of base (24).

The tripeptide  $[^3\text{H}]\text{Val-Leu-Ser}$  was synthesized by the Protein and Nucleic Acids Chemical Laboratory, University of Leicester (Leicester, UK).  $[^3\text{H}]\text{Valine}$  (98% atom enrichment) was supplied by Cambridge Isotope Laboratories (Andover, MA).

### Preparation of standards

*N*-(2-Cyanoethyl)- $[^3\text{H}]\text{Val-Leu-Ser}$ . The tripeptide  $[^3\text{H}]\text{Val-Leu-Ser}$  (38 mg, 0.12 mmol) was dissolved in water (0.25 ml). Sodium hydroxide (1 N, 0.24 ml, 0.24 mmol) and ACN (20  $\mu\text{l}$ , 0.30 mmol) were added. The reaction mixture was left at room temperature for 48 h and then acidified with HCl (2 N, 0.12 ml) to stop the reaction and the product was purified by preparative HPLC (23).

*Globin adducted with ACN.* Fresh erythrocytes (7.5 ml) were washed three times with isotonic saline and incubated with ACN (30  $\mu\text{l}$ ) for 20 h. The cells were then lysed with deionized water and cell debris removed by centrifugation (20 000 g). The globin was precipitated from the supernatant with 1% HCl in acetone. The protein was then washed successively with 1% HCl in acetone, acetone and diethyl ether and then dried.

### Determination of *N*-(2-cyanoethyl)valine in globin

*Standard adducted globin.* The standard adducted globin (5 mg) was mixed with the internal standard, *N*-(2-cyanoethyl)- $[^3\text{H}]\text{Val-Leu-Ser}$  (40 nmol), and submitted to hydrolysis in HCl (1 ml, 6 N) at 110°C for 24 h *in vacuo*. The amino acid mixture resulting from the protein hydrolysis was sonicated in an ultrasonic bath with MTBSTFA + 1% TBDMCS (30  $\mu\text{l}$ ) and acetonitrile (30  $\mu\text{l}$ ) at 65°C for 45 min and then heated to 65°C for a further 45 min. The *t*-butyldimethylsilyl (TBDMSi) derivatives produced were analysed by GC-MS using a VG Trio quadrupole mass spectrometer coupled to an HP5890 Series II gas chromatograph. Samples were injected splitless onto a 30 m  $\times$  0.32 mm Rtx-1701 column supplied by Restek Corp. (Bellefonte, PA). The initial oven temperature was 100°C for 1 min, followed by a temperature ramp at 30°C/min to 270°C. The mass spectrometer was operated in the electron impact ionization mode and selective ion monitoring was carried out of the ion  $m/z$  258 for the TBDMSi derivative of *N*-(2-carboxyethyl)valine and  $m/z$  266 for the corresponding ion from the derivatized deuterated analogue. Quantitation was achieved by reference to a calibration line which was established for a series of samples containing 5 mg control globin, the internal standard *N*-(2-cyanoethyl)- $[^3\text{H}]\text{Val-Leu-Ser}$  (40 nmol) and varying amounts of CEV (0–106 nmol). These samples were hydrolysed and derivatized in the same way as the standard adducted globin (above).

*Modified Edman degradation procedure.* Globin was prepared from human or rat erythrocytes by the procedure described above. Samples of 50 mg were dissolved in formamide to which was added *N*-(2-cyanoethyl)- $[^3\text{H}]\text{Val-Leu-Ser}$  (200 pmol). PFPITC (10  $\mu\text{l}$ ) was added to the samples, which were shaken overnight at room temperature and then for 90 min at 40°C. The resulting CEV-PFPTH was isolated by Sep-Pak  $\text{C}_{18}$  cartridge chromatography. After the sample was applied to the column it was washed with water (4 ml) and hexane (1 ml) and the CEV-PFPTH derivative was then eluted with ethyl

acetate (4 ml). The product was evaporated and the residue redissolved in toluene (2 ml) and washed with  $\text{Na}_2\text{CO}_3$  (0.1 M, 1 ml) and water (1 ml). The toluene extract was then evaporated to dryness and the residue dissolved in acetonitrile (30  $\mu\text{l}$ ) for GC-MS analysis. The GC-MS procedure was similar to that used for *N*-(2-carboxyethyl)valine (above) with the exception that a 30 m  $\times$  0.32 mm Rtx-5 column was used with a temperature programme of an initial temperature of 100°C for 1 min followed by a temperature ramp to 295°C at 30°C/min. The selected ions monitored were the molecular ion of the derivatized adduct at *m/z* 377 and the corresponding ion(s) at *m/z* 384 and/or 385 for the deuterated internal standard.

A calibration line for the quantitation was established for each batch of samples analysed. Aliquots of control globin (50 mg) were mixed with the internal standard peptide (200 pmol) and with a range of amounts of the standard: unlabelled adducted globin. The range of CEV concentrations for the calibration line was adjusted according to the type of samples being analysed: for ACN-exposed workers the amount of CEV in the globin added varied from 0 to 346 pmol, for samples from mothers and their newborns from 0 to 43 pmol and for ACN-dosed rats from 0 to 173 pmol.

#### Treatment of rats with ACN

Eighteen female Fischer 344 rats (body wt 180–196 g) were divided into six groups and to each an oral dose (0, 1, 3, 5, 7 or 10 mg/kg) of ACN was administered by gavage. Blood was collected after 28 h by cardiac puncture, with a heparinized syringe, under ether anaesthesia. The blood was centrifuged (900 g) and the erythrocytes washed three times with phosphate-buffered saline. Water (3 vol.) and phosphate buffer (0.67 M, pH 6.5, 4 vol.) were added to the washed cells. The globin was prepared from this lysate by the procedure described above for the standard globin adducted with ACN.

#### Human sample collection

Blood samples were collected from workers in a polymerization plant where occupational exposure to ACN occurred. These workers were from either the continuous polymerization part of the factory (CP) or were maintenance mechanics (MM). The exposure of the latter group of workers was during repair and cleaning of the polymerization reactors. Active and passive smoking status was established by questionnaire; only non-smoking subjects were included in this study (seven CP and nine MM). Eleven control subjects who were office workers in the same factory were also sampled. Globin was prepared from all haemoglobin solutions employing the same procedure as was used for the animal blood samples.

Blood samples from smoking and non-smoking mothers and their newborns were available from a previous study (25).

## Results

Quantitation of N-terminal CEV was achieved using electron impact selected ion monitoring of the molecular ion ( $M^{+}$ ) of CEV-PFPTH, liberated following a modified Edman degradation of globin. In order to establish calibration lines it was necessary to prepare both an unlabelled ACN adduct of globin and an internal standard, which was the ACN-adducted tripeptide  $[^2\text{H}_3]\text{Val-Leu-Ser}$ . The structure of the latter was determined by NMR and electrospray tandem mass spectrometry (23). The content of CEV in the unlabelled adduct of ACN with globin was determined by GC-MS selected ion monitoring of a derivatized acidic hydrolysate using *N*-(2-cyanoethyl)- $[^2\text{H}_3]\text{Val-Leu-Ser}$  as internal standard. During protein hydrolysis, CEV in globin and  $[^2\text{H}_3]\text{CEV}$  in the internal standard peptide were converted to *N*-(2-carboxyethyl)valine and *N*-(2-carboxyethyl)- $[^2\text{H}_3]\text{valine}$  respectively. Calibration lines were established using control globin mixed with varying amounts of authentic CEV and *N*-(2-cyanoethyl)- $[^2\text{H}_3]\text{Val-Leu-Ser}$ . The amount of CEV in the standard adducted globin was determined to be 18 nmol/mg globin [assuming quantitative conversion of CEV in the globin to *N*-(2-carboxyethyl)valine during the acidic hydrolysis].

Using the above two standards it was then possible to determine the amount of N-terminal CEV in globin using the modified Edman technique. A calibration line was constructed with each batch of analyses using a fixed amount of *N*-(2-cyanoethyl)- $[^2\text{H}_3]\text{Val-Leu-Ser}$  and varying amounts of the

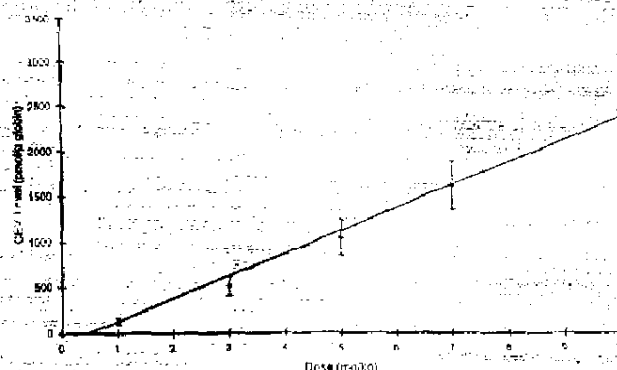


Fig. 1. Dose-response curve for the production of CEV in rats treated with ACN (oral dose, 0–10 mg/kg). Values are the mean  $\pm$  SD for three rats.

Table 1. CEV adduct levels in the globin of ACN-exposed workers, local control workers, smoking mothers and their offspring.

	No. of subjects	Range (pmol/g globin)	Mean $\pm$ SD (pmol/g globin)
Exposed workers (MM)	9	93.9–5746	1984 $\pm$ 2066
Exposed workers (CP)	7	635–4604	2276 $\pm$ 1338
Control workers	11	8.5–70.5	31.1 $\pm$ 18.5
Smoking mothers	13	92.5–373	217 $\pm$ 85.1
Babies from smoking mothers	13	34.6–211	99.5 $\pm$ 53.8

unlabelled adduct of ACN with globin. The ions chosen for the selected ion monitoring were selected on the basis of electron impact mass spectral scans of the unlabelled and labelled derivatives. The scan of the unlabelled CEV-PFPTH derivative showed an intense molecular ion at *m/z* 377, whereas that of the derivative from *N*-(2-cyanoethyl)- $[^2\text{H}_3]\text{Val-Leu-Ser}$  showed both *m/z* 385 and *m/z* 384, in the approximate ratio 1:3.3. On most occasions *m/z* 384 was used to monitor the deuterated internal standard. Similar results were obtained when *m/z* 385 was used. An example of a calibration line (*m/z* 377 and *m/z* 384) suitable for determination of CEV in the range 0–350 pmol gave the relationship  $y = 0.0028x + 0.0041$  ( $r^2 = 0.998$ ). The limit of sensitivity of the assay was 1 pmol CEV/g globin.

The method was first validated using globin samples from ACN-treated rats. Gavage doses up to 10 mg/kg yielded CEV in globin at concentrations up to 2.1 nmol/g globin and the dose-response relationship is shown in Figure 1. The curve appears to be slightly upwardly convex, but close to linear. The linear regression line is  $y = 248.1x - 118.4$  ( $r^2 = 0.991$ ), where  $y$  is the concentration of CEV in pmol/g globin and  $x$  is the dose in mg/kg. The amount of CEV in the globin of untreated rats, if present, was below the limit of detection.

Non-smoking workers ( $n = 16$ ) who were exposed occupationally to ACN in a factory where ACN polymerization was carried out were then analysed for CEV in globin. Control subjects ( $n = 11$ ) working in the same factory, but doing office work, were used for comparison. The CEV levels in all of the exposed workers were readily detectable, being in the range 93.9–5746 pmol/g globin (Table 1 and Figure 2). A representative selected ion monitoring trace is depicted in Figure 3. No significant difference was seen between the CP and MM workers, whose mean CEV levels were  $2276 \pm$  SD

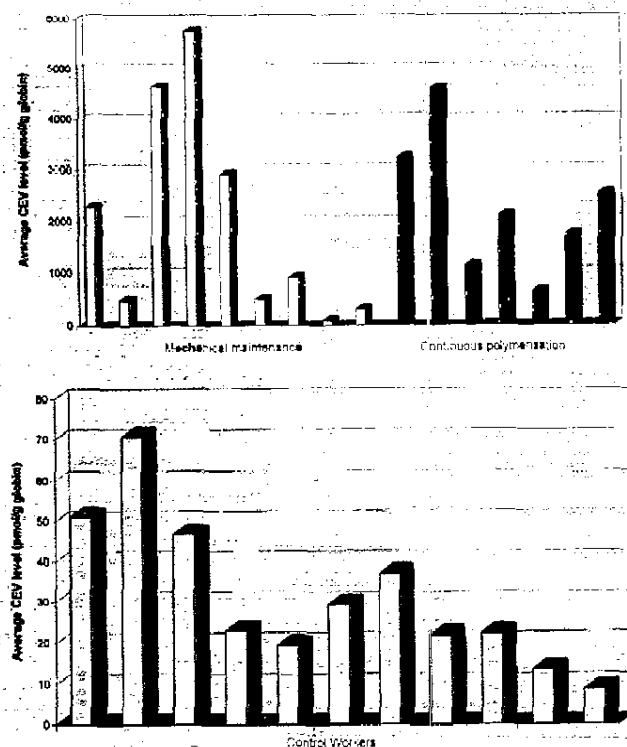


Fig. 2. (a) CEV adduct levels (pmol/g) in the globin of workers dealing with ACN in a polymerization plant in two areas: continuous polymerization (CP) and mechanical maintenance (MM). (b) CEV adduct levels (pmol/g) in the globin of control workers in the same factory (office workers).

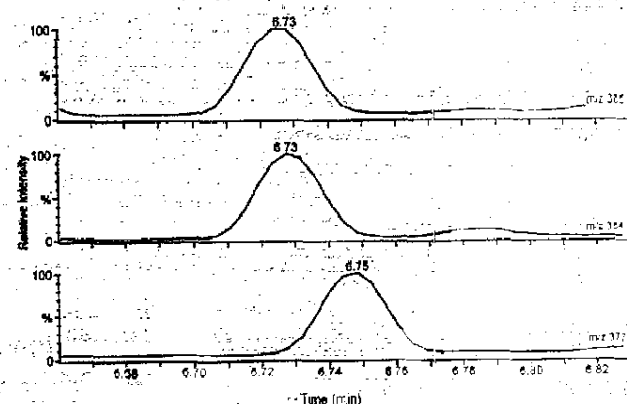


Fig. 3. A representative selective ion monitoring GC-MS trace ( $m/z$  377, 384 and 385) from analysis of the CEV adduct in the haemoglobin of a worker exposed to ACN.

1338 and  $1984 \pm \text{SD } 2066$  pmol/g globin respectively. The levels detected were significantly higher ( $P < 0.001$ ) than those found in the control individuals. In the latter population, the range of values was 8.5–70.5 pmol/g globin.

The concentration of N-terminal CEV in globin from 13 smoking mothers and their newborn babies was then determined. Twelve of these mothers had previously been monitored for exposure to ethylene oxide derived from constituents of tobacco smoke by measurement of N-terminal N-(2-hydroxyethyl)valine (HEV) in globin (25). Globins of 10 non-smoking

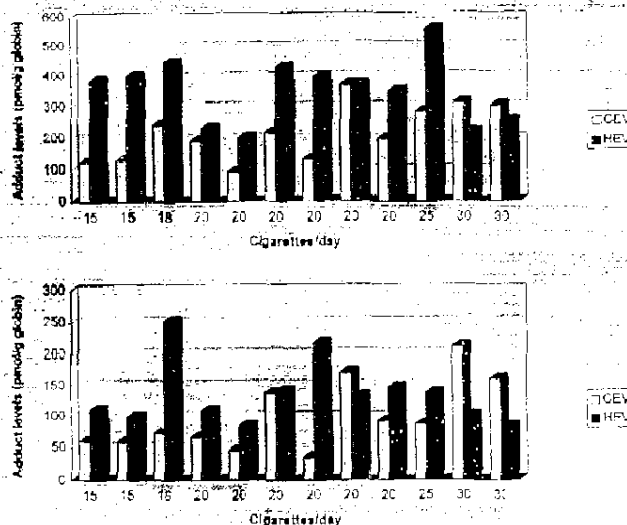


Fig. 4. The content of CEV and HEV (pmol/g) in the globin of smoking mothers (a) and their newborns (b).

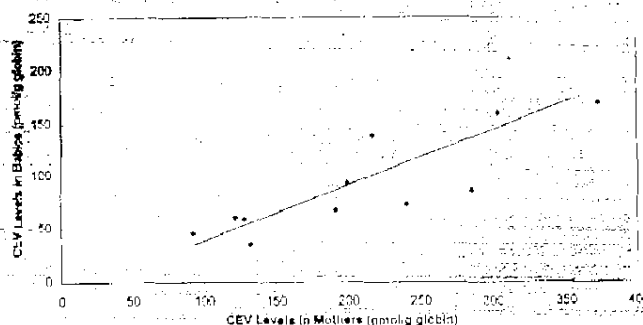


Fig. 5. Correlation between CEV globin adducts levels in smoking mothers and their newborn babies.

mothers and their newborn babies were analysed as controls. The range of CEV levels in the smoking mothers' globin was 92.5–373 pmol/g globin and in their newborn babies 34.6–211 pmol/g globin (Table I). The CEV concentrations in the globin of control mothers and their newborn babies were below the limit of detection.

The CEV levels in the globin of all but two mothers and all but three babies were lower than the HEV levels that were previously determined in the same globin samples (Figure 4). There was no statistically significant relationship between CEV and HEV in either the mothers' or the babies' samples. Additionally, neither of these correlations was statistically significant at  $P = 0.05$  when the background adduct levels were subtracted from the HEV levels. However, in the samples from the smoking mothers there was a significant relationship between the number of cigarettes smoked per day and the CEV levels in globin ( $r = 0.633$ ,  $P = 0.020$ ). There was also a significant correlation ( $r = 0.691$ ,  $P = 0.009$ ) between the number of cigarettes smoked by the mother and the CEV levels in the babies' globin. A highly significant correlation ( $r = 0.831$ ,  $P = 0.001$ ) existed between the CEV levels in smoking mothers' globin and that in the globin of their newborns (Figure 5).

## Discussion

Our analytical approach differs from that of Osterman-Golkar *et al.* (20) and Bergmark *et al.* (21) in that we use the novel internal standard *N*-(2-cyanoethyl)-[ $^2\text{H}_8$ ]Val-Leu-Ser, which replicates the N-terminal sequence of haemoglobin, and electron impact selected ion monitoring as opposed to negative ion chemical ionization monitoring. Also, the standard employed here has a high stable isotope content, [ $^2\text{H}_8$ ], which is advantageous for mass spectral selected ion monitoring and greater than could be achievable by the use of an adduct containing stable isotope-labelled ACN. [The [ $^2\text{H}_8$ ]Val-Leu-Ser used for synthesis of the internal standard for CEV determination may similarly be used for preparing [ $^2\text{H}_8$ ] standards for determining adducts of other compounds that react with haemoglobin N-terminal valine (24).] The limit of detection of our assay is 1 pmol CEV/g globin, which has been shown to be sufficient for quantitating CEV in globin from exposed animals and workers, smokers, babies of smokers and some control non-smoking individuals.

The dose-response relationship for the production of CEV in rats treated with ACN by gavage was close to linear over the range of doses 0–10 mg/kg. ACN at 1 mg/kg yielded 248 pmol CEV/g globin based on a linear extrapolation. In comparison with adduct levels that we have previously observed for other genotoxic agents, this level of alkylation is relatively abundant. Thus, for example, styrene oxide was shown by us to produce in rats only ~60 pmol N-terminal valine adduct/g globin after an i.p. dose of 50 mg/kg (26). Monitoring of this adduct in human samples has also successfully been achieved (27).

For the exposed workers in our study, the levels of CEV in globin ( $1984 \pm \text{SD } 2066$  pmol/g globin for the MM group and  $2276 \pm \text{SD } 1338$  pmol/g globin for the CP group) were significantly higher than those of the control workers in the same factory ( $31.1 \pm 18.5$  pmol/g globin;  $P < 0.01$  following a one-way analysis of variance with a logarithmic transformation of the data). The range of exposed values was 93.9–5746 pmol/g globin for the MM group and 635–4604 pmol/g globin for the CP group. This may be compared with the CEV adduct levels observed by Bergmark *et al.* (21) in a group of 41 workers who were involved in the synthesis of acrylamide by catalytic hydration of acrylonitrile in a factory in the People's Republic of China. The values observed in that study had a higher maximum and were in the range 20–66 000 pmol/g globin. It is apparent, however, that the exposure in the Portuguese group was not continuously at a high level. Of interest was the fact that the control workers working in offices in the Portuguese factory had measurable levels of CEV in their globin (8.5–70.5 pmol/g globin). These values were much higher than those in the globin of non-smoking mothers, which were undetectable. This may indicate that this control population was also exposed environmentally to ACN, either through the air (e.g. environmental tobacco smoke or ACN from the nearby occupational source) or through drinking water.

The CEV levels that we observed in the smoking population averaged  $217 \pm 85.1$  pmol/g globin and were in agreement with the values observed by Osterman-Golkar *et al.* in a much smaller population (20). In our study, significant correlations were found between the number of cigarettes smoked per day by the mother and the CEV levels in both the mothers' and the newborns' globin. In all but one of these samples, the HEV levels had previously been determined (25). However,

in that study no correlation was found between the number of cigarettes smoked by the mother and the HEV levels, either in the mothers' or in the babies' globin. The source of HEV in globin of cigarette smokers is not entirely clear. It is possible that some of it is derived from the small proportion of ethylene oxide in cigarette smoke, but that a larger proportion may be derived from ethylene, which is metabolized *in vivo* to ethylene oxide. A further source of HEV in control non-smoking subjects is thought to be endogenous ethylene (28) and it is presumably also possible that cigarette smoking may perturb and/or accentuate the processes that generate this ethylene. In view of the likelihood of interindividual variation in both the oxidative metabolism of ethylene and in endogenous ethylene production it seems apparent that HEV may not be as good a marker of exposure to cigarette smoke as CEV. The absence of significant correlations between CEV and HEV in mothers or children might be due to variations in the rate of detoxification, particularly of ethylene oxide. Even after subtraction of the background level of HEV, no significant correlation was found. Calculations of statistical power (29) show that with a sample size of 12 there would have been a 98% chance of detecting a correlation of  $\pm 0.8$  using a two-sided test with  $\alpha = 0.05$ . Both biomarkers will, of course, measure the biologically active concentration of the active electrophile that reaches red blood cells, which may be more relevant for risk assessment than cigarette smoke exposure.

Also of relevance to risk estimation from ACN exposure is production of the mutagenic metabolite CEO, which would also be expected to form adducts with DNA and proteins. The PPTH derivative of the potential valine adduct of CEO, *N*-(2-cyano-2-hydroxyethyl)valine has recently been synthesized by Lawrence (24), but this is unstable in the underivatized form to analysis by GC. Attempts are currently in progress to develop an assay for this compound.

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